

## Ultrasonographic Evaluation of the Kidney in 5/6 Nephrectomized Rats: Correlation with Biochemical and Histopathological Findings

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**Abstract.** *Aim: To characterize the kidney alterations associated with renal mass reduction by two-dimensional ultrasound and assess the correlation of the ultrasonographic parameters with the histological and biochemical findings. Materials and Methods: Rats were divided into two groups: sham-operated animals (n=13), and animals which underwent renal mass reduction (RMR) through 5/6 nephrectomy (n=14). Renal length, renal thickness, cortical thickness, medullary length and echogenicity of the kidneys were evaluated by ultrasonography at 3 and 6 months after the RMR. Results: Except for the renal length, the renal dimensions at 3 and 6 months were significantly higher in the RMR group when compared to the sham-operated group ( $p<0.05$ ). Furthermore, the cortical and medullary echogenicity was significantly higher in the RMR group when compared to the sham-operated group ( $p<0.05$ ). A significant correlation was observed between the plasma creatinine level and the renal length 3 months after RMR ( $r=-0.612$ ,  $p=0.045$ ). Conclusion: These data support future application of ultrasonography for monitoring the progression of renal damage in chronic studies with the 5/6 nephrectomy model.*

Chronic kidney disease (CKD) is a major public health problem affecting approximately 8-16% of the population worldwide (1). Animal models are important tools in studying CKD. The renal mass reduction (RMR) animal model has

been a mainstay of studies of progressive CKD, mainly in studying the pathogenesis and novel therapy of kidney diseases (2, 3). After 5/6 nephrectomy, animals develop several kidney alterations, such as glomerular hypertrophy, tubular atrophy, glomerular and interstitial fibrosis, and renal insufficiency (4-7).

In the model of RMR, assessing renal dysfunction and histopathological alterations is of great importance in the evaluation of new therapeutic approaches for CKD (8, 9). In humans, renal biopsy has been advocated in patients with CKD (10, 11). However, its invasive nature and the need for serial biopsies in order to monitor progression of the disease would expose animal models to repeated risks and is not practical. Therefore, certain biochemical markers, such as blood urea nitrogen (BUN), creatinine, proteinuria and creatinine clearance, are typically used to assess renal dysfunction in this model of CKD (8, 9). However, laboratory detections primarily demonstrate functional abnormalities of the kidneys.

Two-dimensional ultrasonography (US), an established non-invasive imaging technique, is appealing because of its simplicity, low cost, non-invasiveness and safety, widespread availability and non-nephrotoxicity (12-15). Studies have demonstrated that transabdominal renal US is both repeatable and reproducible (16, 17) and it is routinely performed in animals with suspected renal disease (18). The distinction between normal kidney and kidney with chronic damage by two-dimensional US imaging is currently based on morphological changes, including in size and parenchymal echogenicity. Increased cortical or cortical and medullary echogenicity is reported to be one of the most common signs of chronic or acute kidney disease in veterinary medicine (18). In human medicine, a high correlation between cortical echogenicity and glomerular sclerosis, tubular atrophy, interstitial fibrosis and interstitial inflammation has been

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described (19). Consequently, US may be a potential tool for monitoring the progression of renal damage in the RMR model.

The aims of the current study were to characterize kidney alterations associated with RMR in Wistar rats by two-dimensional US, and compare renal dimensions obtained by this method with post-mortem measurements. We also assessed the correlation of the US parameters with the histological and biochemical changes during the progression of kidney disease in this model.

## Materials and Methods

**Animals.** Thirty male Wistar rats (*Rattus norvegicus*) of 4 weeks of age (112-157 g body weight) were acquired from Harlan Interfauna Inc. (Barcelona, Spain). Animals were housed in filter-capped polycarbonate cages (Tecniplast, Buguggiate, Italy) with corn cob for bedding (Mucedola, Milan, Italy) at the University of Trás-os-Montes and Alto Douro. All cages were maintained on a 12/12-h light-dark cycle in a ventilated room under controlled conditions of temperature ( $23 \pm 2^\circ\text{C}$ ) and relative humidity ( $50 \pm 10\%$ ). Cages were cleaned once per week and water was changed weekly. Animals had *ad libitum* access to a basic standard diet (4RF21®; Mucedola) and bottled water throughout the study.

**Surgical procedure and experimental groups.** After a period of acclimation of 7 weeks, each rat was weighed (359 to 402 g) and anesthetized by intraperitoneal administration of ketamine (Imalgene® 1000, 70 mg/kg; Merial S.A.S., Lyon, France) and xylazine (Rompum® 2%, 10 mg/kg; Bayer S.A., Kiel, Germany). RMR was performed in 17 rats. The animals were subjected to right nephrectomy and surgical removal of the left kidney poles (8). A sham operation was performed on 13 additional rats as a non-nephrectomized control (sham-operated; SO). One week after surgery, surviving animals were randomly divided into SO ( $n=13$ ) and RMR ( $n=14$ ) groups. All procedures followed the European (European Directive 2010/63/EU) and National (Decree-Law 113/2013) legislation on the protection of the animals used for scientific purposes.

**Ultrasonographic evaluation.** At 3 and 6 months after the surgical procedure, the left kidney of each animal was evaluated by ultrasonography using two-dimensional US (B mode). For this, the left flank of each animal was shaved using a machine clipper (AESCULAP® GT420 Isis; Aesculap Inc., Center Valley, PA, USA). Animals were restrained in prone position without anesthesia and sagittal US images of the kidney were obtained under the same conditions in all animals using acoustic gel (Aquasonic®; Parker Laboratories Inc., NJ, USA) and a real time scanner (LogiqP6®; General Electric Healthcare, WI, USA) with a 10 MHz linear probe. The images were recorded for subsequent analysis. For each image, the renal length was measured as the greatest pole to pole distance and the renal thickness was measured as the distance between the renal capsule and pelvis, perpendicular to the renal length. The cortical thickness was measured as the distance from the renal capsule to the base of medullary pyramid. Additionally, the medullary length was measured perpendicular to the medullary thickness (Figure 1). The mean cortical and medullary echogenicity was evaluated; for this, an area of approximately  $1\text{ cm}^2$  was evaluated

in cortex and medulla using the standard histogram function of Adobe Photoshop CC (Adobe systems Inc., San Jose, CA, USA) (Figure 2).

**Sample collection.** Blood and urine samples were collected at 3 and 6 months. For urine sample collection, animals were placed in metabolic cages with free access to food and water for 2 days in order to accustom them to isolation conditions. Twenty-four-hour urine samples were collected during the next 2 days to determine urine volume. Subsequently, urine samples were centrifuged at  $1500 \times g$  for 15 min and stored at  $-20^\circ\text{C}$  for determination of creatinine and protein concentration. Blood samples ( $150\text{ }\mu\text{l}$  per animal) were obtained from a cut in the tail tip directly into heparinized capillary tubes. The tubes were centrifuged at  $1,500 \times g$  for 15 min, plasma was stored at  $-20^\circ\text{C}$  to determine creatinine concentration. Glomerular filtration rate was estimated from the creatinine clearance, which was calculated by employing a standard formula (urinary creatinine  $\times$  urinary volume/serum creatinine).

**Animal sacrifice.** Six months after surgery, all animals were anesthetized with isoflurane and humanely sacrificed. A complete necropsy was performed; the remnant kidney from RMR rats or both kidneys from rats of the SO group were removed, measured with a ruler after sagittal sectioning (anatomopathological measurement) and immersed in 10% buffered formalin for 24 h.

**Histopathology.** After fixation, the kidneys from all animals were cut and embedded in paraffin blocks. Subsequently,  $2\text{-}\mu\text{m}$  sections were obtained and mounted on glass slides. For morphometric evaluation, sections were stained with hematoxylin and eosin (H&E), Reticulin and Masson's trichrome and evaluated blindly by two pathologists under a light microscope. Renal fibrosis was evaluated and scored as reported previously by Asaba *et al.* (20).

**Statistical analysis.** A descriptive analysis was performed for all variables included in the study. Data were statistically analyzed with SPSS® (version 23 for Windows; IBM Corp., Armonk, NY, USA) using the independent and paired *t*-test. Spearman correlation was used to assess the correlation between US, histological and biochemical data. Data are presented as the mean  $\pm$  standard deviation (S.D.). A *p*-value lower than 0.05 was considered statistically significant.

## Results

**Animals.** Two animals from the RMR group died (9 and 18 weeks, respectively) after surgery. The data from these animals were not included in the study and the size of the group was reduced to 12 animals in the RMR group. Due to the fact that the US examination was performed in awake animals, adequate images of the kidney could not be obtained in one animal from each experimental group. In this way, only data from 12 animals of the SO group and 11 animals of the RMR group are presented.

**Renal measurements by US at 3 and 6 months of the protocol.** Statistically significant differences were observed between SO and RMR groups for renal thickness and

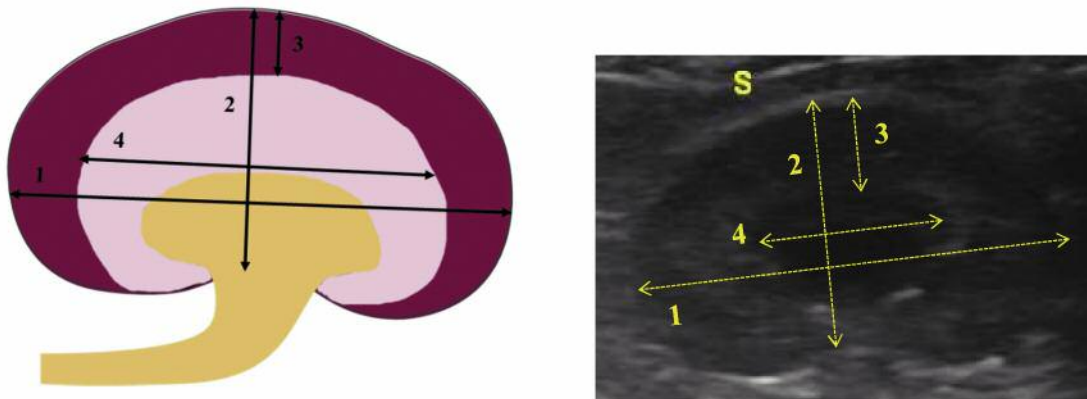


Figure 1. Schematic (A) and ultrasonographic (B) imaging of a normal rat kidney (sham-operated group) in sagittal section (S) (1: renal length; 2: renal thickness; 3: renal cortical thickness; 4: renal medullary length).

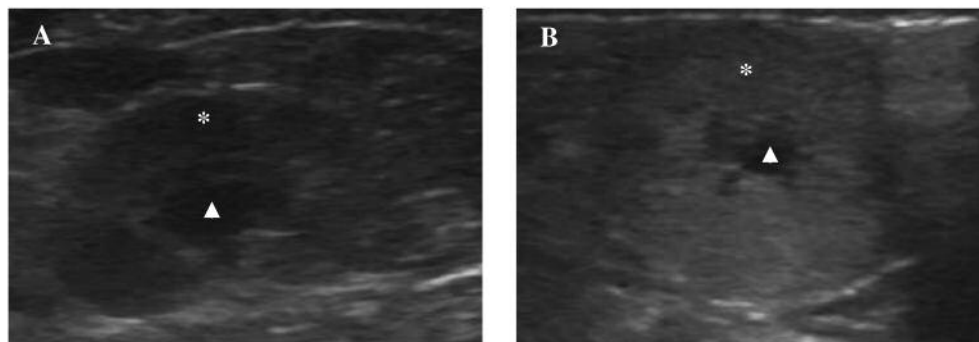


Figure 2. Ultrasonographic images of the kidney in sham-operated (A) and renal mass reduction (B) groups 6 months after 5/6 nephrectomy, revealing the renal cortex (asterisk) and renal medulla (arrowhead).

medullary length at 3 months of the protocol, and for renal thickness, cortical thickness and medullary length at 6 months of the protocol ( $p < 0.05$ ) (Table I). For each experimental group, the renal measurements were higher at 6 months when compared with those at 3 months of the protocol. However, statistically significant differences were only found for renal length in both SO and RMR groups, and for cortical thickness in the RMR group ( $p < 0.05$ ) (Table I).

*Comparison between renal measurements using a ruler and by US at 6 months of the protocol.* The renal measurements using a ruler were lower when compared to renal measurements obtained by US (Table II). Statistically significant differences were found between US and anatomopathological measurements of renal length in the RMR group ( $p < 0.05$ ) and of renal thickness in both SO and RMR groups ( $p < 0.05$ ) (Table II).

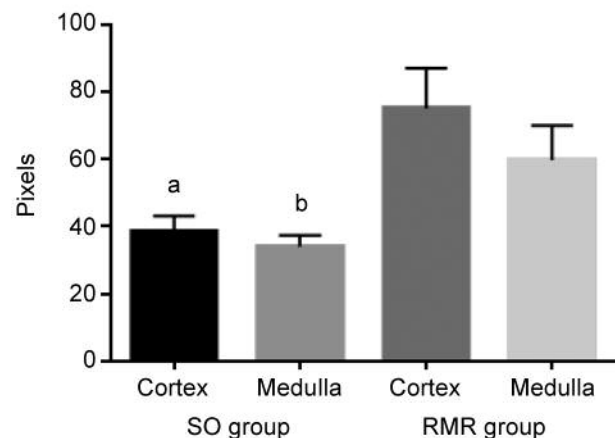


Figure 3. Evaluation of echogenicity in renal cortex and medulla in sham-operated (SO) and renal mass reduction (RMR) groups 6 months after the surgery. Significantly different at  $a p < 0.05$  versus cortex in the RMR group,  $b p < 0.05$  versus medulla in RMR group.

Table I. Measurement of renal dimensions (mean±S.D.) by ultrasonography at 3 and 6 months after renal mass reduction (RMR).

Group	Ultrasonographic measurement (cm)							
	Renal length		Renal thickness		Cortical thickness		Medullary length	
	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months
SO (n=12)	1.87±0.08 <sup>a</sup>	1.96±0.16 <sup>b</sup>	1.15±0.07 <sup>a</sup>	1.18±0.11 <sup>a</sup>	0.39±0.04 <sup>a</sup>	0.39±0.05 <sup>a</sup>	0.75±0.11 <sup>a</sup>	0.80±0.09 <sup>a</sup>
RMR (n=11)	1.83±0.18 <sup>a</sup>	2.06±0.34 <sup>b</sup>	1.44±0.16 <sup>b</sup>	1.57±0.25 <sup>b</sup>	0.48±0.12 <sup>a</sup>	0.58±0.17 <sup>b</sup>	0.96±0.21 <sup>b</sup>	0.99±0.18 <sup>b</sup>

SO: Sham-operated rats. For each parameter, different letters correspond to statistically significantly different values ( $p<0.05$ ).

**Renal echogenicity.** The cortical and medullary echogenicity was evaluated in US images from both experimental groups at 6 months of the experimental protocol. The cortical and medullary echogenicity was significantly higher in the RMR group when compared with the SO group ( $p<0.05$ ) (Figure 3).

**Correlation of ultrasonographic parameters with the histological and biochemical parameters in RMR group.** Only one statistically significant correlation was observed and was between the plasma creatinine and the renal length 3 months after RMR ( $r=-0.612$ ,  $p=0.045$ ). However, moderate correlations were observed between: creatinine clearance and renal length ( $r=0.530$ ,  $p=0.094$ ); and proteinuria and renal thickness ( $r=0.451$ ,  $p=0.164$ ), 3 months after RMR. Additionally, a moderate correlation was observed between glomerulosclerosis and cortical thickness ( $r=0.461$ ,  $p=0.131$ ) 6 months after RMR (Table III).

## Discussion

The model of 5/6 nephrectomy in rats is a widely studied animal model of chronic renal failure that is close enough to the pathophysiological characteristics of human CKD (21). Hence, this model is used to study different aspects of CKD, namely to assess the potential renoprotective effects of new drugs (8, 9). Detection of renal damage and fibrosis in this model is very important in chronic studies and finding a non-invasive means of its detection would provide tremendous utility in experimental studies.

US is a real-time imaging modality commonly used in *in vivo* evaluation of the kidney, and is a relatively cheap, non-invasive and non-nephrotoxic modality (12). Therefore, we applied this imaging modality in conscious rats subjected to 5/6 nephrectomy in order to follow-up the progression of renal damage in a chronic experimental study (6 months). To our knowledge, no studies have yet been performed in order to detect alterations in renal size and thickness and echogenicity of the renal cortex and medulla by US in this model.

Table II. Measurement of renal dimensions (mean±S.D.) in sham-operated (SO) and renal mass reduction (RMR) groups by ultrasonography and anatomopathologically by ruler at the end of the experimental protocol.

Group	Renal length (cm)		Renal thickness (cm)	
	Ultrasound	Ruler	Ultrasound	Ruler
SO (n=12)	1.96±0.16 <sup>a</sup>	1.91±0.24 <sup>a</sup>	1.18±0.11 <sup>a</sup>	1.10±0.10 <sup>b</sup>
RMR (n=11)	2.06±0.34 <sup>a</sup>	1.52±0.23 <sup>b</sup>	1.57±0.25 <sup>c</sup>	1.33±0.17 <sup>d</sup>

For each parameter, different letters correspond to statistically significantly different values ( $p<0.05$ ).

Histopathological studies of renal tissue after renal mass reduction revealed the presence of three phases: I: fast hypertrophic phase (2 to 4 weeks after RMR); II: phase with minimal histological changes (4 to 10 weeks); and III: development of glomerular sclerosis and tubulointerstitial fibrosis (after 10 weeks) (22). Therefore, we started the evaluation of the kidneys by US 3 months after the 5/6 nephrectomy.

In general, the kidney dimensions (renal length, renal thickness, cortical thickness and medullary length) were higher in the RMR group when compared with the SO group at 3 and 6 months of the protocol. These results are in accordance with what would be expected, since compensatory kidney hypertrophy in the animals of the RMR group as a consequence of renal mass reduction was predictable (23). In both groups, the renal dimensions were higher at 6 months of the experimental protocol when compared with the same dimensions at 3 months of the protocol, which might be attributable to normal animal growth because renal size changes with age (24).

At the end of the experimental protocol, kidney dimensions were also measured using a ruler (anatomopathological dimensions). All dimensions of the kidney measured in this way were lower when compared to US dimensions. However, this may be due to the lack of blood supply that occurs immediately after the excision of the kidney from the animals, with a consequent reduction in

Table III. Spearman correlation coefficients between ultrasonographic measurements and biochemical and histological parameters, at 3 months and at the end of the experimental protocol (6 months) in the renal mass reduction group (n=11).

Group	Renal length		Renal thickness		Cortical thickness		Medullary length		Cortical echogenicity		Medullary echogenicity	
	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months
Proteinuria	0.233	0.102	0.451	0.158	-0.082	0.039	-0.136	0.155	nd	-0.357	nd	0.214
Plasma creatinine	-0.612*	-0.025	-0.319	-0.032	-0.169	0.155	-0.382	-0.098	nd	-0.071	nd	-0.321
Creatinine clearance	0.530	0.189	0.282	-0.035	0.174	-0.272	0.018	0.081	nd	0.393	nd	0.500
Glomerulosclerosis	nd	0.031	nd	0.207	nd	0.461	nd	0.199	nd	0.418	nd	-0.120
Interstitial fibrosis	nd	-0.140	nd	0.028	nd	0.056	nd	-0.112	nd	×	nd	×

\*Statistically significant at  $p < 0.05$ . ×: It was only possible to obtain three results for interstitial fibrosis hence it was not possible to determine the correlation coefficients; nd: not determined.

kidney dimensions. Abershon-butche *et al.* compared renal dimensions in horses obtained using the US method and post-mortem examination. These authors also observed that US technique overestimated the size of the kidneys (25).

The existing US equipment in our laboratory allows the clear differentiation of the cortex and medulla. However, it was not possible to determine cortical and medullary echogenicity at 3 months after RMR, primarily because of a problem with image quality at that time. Hence the cortical and medullary echogenicity was evaluated in both groups only at 6 months after RMR.

According to several researchers, variation of echogenicity may be an indicator of alteration in several tissues (19, 26-28). In the kidney, an increase of echogenicity is considered an indicator of renal fibrosis (29). In the present study, the cortical and medullary echogenicity was significantly higher in the RMR group when compared with the SO group. These results seem to indicate that the echogenicity evaluated in either cortex or medulla is a good sensitive marker of fibrosis in the 5/6 nephrectomy model.

Previous work performed both in humans and animals with renal disease have determined the kidney dimensions by US in order to compare the data with histopathological findings (30-32). In this study we correlated US data with histopathological and also with biochemical findings. Although Moghazi *et al.* observed a significant correlation between echogenicity and histopathological data in humans (19), we did not observe a significant correlation between these two variables in Wistar rats. In this study, we observed only a statistically significant correlation between renal length and plasma creatinine 3 months after RMR. However, moderate correlations were observed between the creatinine clearance and the renal length; and proteinuria and the renal thickness, 3 months after RMR. Additionally, a moderate correlation was observed between the glomerulosclerosis and the cortical thickness 6 months after RMR. Contrarily to what was observed in humans (19, 31), the findings of this study

seems to indicate that the US parameters are more associated with biochemical parameters of renal function than with histological alterations. However, an explanation for our results may be the low number of animals used in this study. Moreover, it would also have been important to evaluate other US parameters, such as resistive index, pulsatility index, peak systolic velocity and end-diastolic velocity, in order to better characterize the changes in kidney during CKD progression.

This preliminary study demonstrates that it is possible to image kidney in conscious rats by two-dimensional US and supports future application of this technique for monitoring the progression of renal damage in chronic studies with the RMR model, where anesthetic application is not possible. Unlike other procedures that can only be determined after the animals' sacrifice, the US evaluation of the kidney may be performed more than once, at any time during the study.

## Conflicts of Interest

Nothing to disclose.

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